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Bone Metastases

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14. ABSTRACT Patients with tumors in the pelvic region frequently receive radiation therapy, and as a result, bystander bone may experience adverse effects. Earlier reports demonstrated that radiation-induced bone loss occurs via increased osteoclast activation in a mouse model. Apoptosis of Osteocytes has been shown to trigger osteoclast progenitor recruitment and differentiation. We hypothesized that radiation induces osteocyte apoptosis leading to increased osteoclast formation and subsequent bone loss. We evaluated the effects of radiation on MLO-Y4 (osteocytes), cell lines. Radiation with 2Gy dose dependently increased the number of trypan blue positive MLO-Y4 cells. We analyzed if the irradiated osteocytes can directly or indirectly stimulate osteoclast formation. We used the conditioned media of irradiated MLO-Y4 cells and treated them on RAW 264.7 cells and also co-cultured MLO-Y4 cells with RAW 264.7 cells and irradiated them using various doses of radiation. We used a single limb irradiation in-vivo model to demonstrate the effects of radiation on the hind limbs of 20 week old female C57/Bl6 mice. There was no evidence that irradiated osteocytes could stimulate osteoclastic activity and osteoclastogenesis, either indirectly (conditioned medium) or directly (co-culture). In the absence of other cell types, irradiation of RAW 264.7 osteoclast precursors already appeared to stimulate osteoclastic activity. We analyzed various bone parameters using microCT and histology (TRAP stain, dynamic bone parameter). We also used TUNEL assay to analyze the amount of apoptosis radiation inflicted on the hind limbs. Radiation decreased the BV/TV, ConnD and DA compared to the non-irradiated tibia. There was also significant increase of TRAP positive osteoclast per bone surface (Oc.N/mmBS) on the irradiated tibia. The TUNEL assays for apoptotic osteocytes revealed significant increase of TUNEL positive osteocytes on the trabecular bone of the irradiated bone. In conclusion, We found no evidence to support that radiation-induced bone loss is mediated via osteocyte apoptosis. Alternatively, direct effects of radiation on osteoclast progenitors and/or indirect effects on bone marrow cells may stimulate osteoclastogenesis and drive radiation-induced bone loss.					
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Patients with tumors in pelvic region (e.g., prostate cancer, cervical cancer) frequently receive radiation therapy to treat the primary tumor (1). Depending on tumor type and stage, ~27 doses of 2Gy are given on consecutive days. Although radiation is effective for treatment of cancer, bystander bone may experience adverse effects, including bone loss and increased fracture risk, particularly at the hip. During the treatment of pelvic soft-tissue tumors, bones of the hip absorb radiation (2). The dose can be substantial; in cervical cancer treatment, approximately half (27 Gy) of the 54 Gy targeted at the tumor will be absorbed by structural components of the hip. This can lead to bone insufficiency resulting in stress fractures, bone fragmentation, and joint dissolution (3,4). The risk of hip fracture, compared to the general population, increases about 11.4-19.7% within 5 years of radiation treatment of cervical, rectal or anal cancer (5-7). This causes considerable morbidity and mortality to the patient during the course of treatment and poses a significant threat to overall patient outcome. It has long been recognized that radiation induces bone loss. Earlier reports showed that radiation could inhibit osteoblast proliferation and survival, which may cause net bone loss. Recently this view was challenged by a group, who showed a marked increase in osteoclast activity after 24 hours (8,9). However, the mechanism of this rapid increase is unknown. It becomes increasingly clear that osteocytes regulate bone turnover, and that regions of osteocyte apoptosis precede increased bone resorption by osteoclasts. This raises the possibility that the apoptotic process may generate a signal used to target osteoclastic bone destruction (10). Recently, it was shown that osteocyte apoptotic bodies stimulate osteoclastogenesis (11). Limited reports in literature showed that radiation resulted in eradication of osteocytes (12,13). In this project, we are studying the effects of radiation on bone cells (osteoblasts, osteocytes, osteoclasts) in *in vitro* models as well as in *in vivo* models of experimental radiation. Our preliminary data indicate that increased bone destruction is associated with more bone metastasis in mouse models of prostate cancer. Thus, radiation to bystander bone may cause bone loss, increase bone resorption, and increase the development and progression of cancer metastasis to bone. The results obtained in this project will increase our understanding of a relatively common, but unstudied area that affects many patients with prostate cancer, and may eventually contribute to implementation of therapeutic interventions that can reduce fracture risk in patients receiving radiation therapy. Finally, the studies will provide new insight into tumor biology and the impact of insults to the bone microenvironment on prostate cancer bone metastasis.

Task 1: Determine time- (6–48 hours) and dose-(0-10Gy) dependent effects of radiation on apoptosis of the osteocyte cell line and freshly isolated osteocytes (vs. osteoblasts) (months 1-8):

a. Perform Apoptosis assays on (TUNEL and Trypan Blue staining, AnnexinV-EGFP/Propidium flow cytometry) (months 2-4)

1. Osteocyte-like cell line, MLO-Y4 (vs. MC3T3-E1 osteoblast cell line)

MLO-Y4 (osteocytes), MC3T3-E1 (osteoblast progenitor) and UMR-106-01 (osteoblast-like/osteosarcoma) cell lines were exposed to doses of 2 to 10 Gy radiation, and cell death and apoptosis were determined by trypan blue exclusion and by caspase-3 activation and annexin-V expression using flow cytometry, respectively.

Radiation with 2Gy significantly increased the number of trypan blue positive MLO-Y4 cells up to 14.9%, 15.5% and 14.6 % after 6, 24 and 48 hours respectively (vs 5.1-7.8% at Co levels), and 10Gy further increased these numbers up to 16.0%, 19.7% and 21.4% (**Figure 1A**). The increases in trypan blue positive UMR-106-01 and MC3T3-E1 cells were most prominent 24 hours after 10Gy radiation (41.5% and 42.1% respectively) (**Figure 1B and C**).

Flow cytometric analysis for apoptosis using various markers (Annexin-V and Caspase-3) yielded similar results. Annexin-V detects the membrane phospholipid phosphatidyl serine (PS) that translocates from inner face of plasma membrane to cell surface soon after initiation of apoptosis (14,15) while Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (16,17). While a marginal induction of apoptosis in MLO-Y4 cells was observed with 2Gy radiation, higher doses of radiation resulted in a much greater induction of apoptosis. At later time points (48hr + 7d), 10Gy radiation induced expression of the apoptotic markers in 50-60% of the MLO-Y4 cells. Similar trends were found for UMR-106-01 and MC3T3-E1 cells (**Figure 2 and 3**).

In conclusion, radiation increases cell death and apoptosis in MLO-Y4 osteocytic cells, in a time and dose dependent fashion

2. Isolated osteocytes (vs. osteoblasts) from mouse calvariae

Animal Usage: 5 balb/c mice; Cells: MLO-Y4, MC3T3-E1 cells + freshly isolated osteocytes and osteoblasts

We were not able to isolate osteocytes from the calvariae of neonatal mice, but instead, we isolated the calvariae from neonatal mice and irradiated them with different doses and time points. We were not able to find significantly more empty lacunae in the H&E sections of irradiated neonatal calvariae. We are planning to detect late phase apoptosis with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and

see if the radiation is sufficient enough to cause DNA damage and hence osteocyte apoptosis in an ex-vivo model.

- ❖ Completion of this task will reveal whether MLO-Y4 osteocytes and freshly isolated osteocytes are sensitive to the effects of radiation. *Collaborator: Dr. Bellido*

Task2: Determine effect of osteocyte apoptosis on osteoclastogenesis (months 6-9)

- a. Establish a new in vitro Osteoclast Assay using RAW264.7 osteoclast progenitor (Corning Inc.) (months 6-7).

A new osteoclast assay has been set up in the lab using the bone mimetic surfaces of Corning ®. Addition of the osteoclastogenic factor RANKL to the RAW264.7 osteoclast progenitors dose-dependently increases the number of TRAP positive osteoclasts and the area of the pits (**Figure 4**). With this experiment, we determined the optimal concentration of RANKL in which the RAW264.7 cells start to differentiate so as to find the basal level of RANKL to add to the irradiated osteocyte conditioned medium. Through this experiment, 5ng/ml RANKL is added as a basal level of osteoclastogenesis that could be further increased or decreased by radiation.

- b. Test osteoclastogenic capability of apoptotic bodies and medium from osteocytes (Task1) (months 8-9).

We collected the conditioned medium including the apoptotic bodies from irradiated and non-irradiated MLO-Y4 cells and added them to a plate seeded with RAW264.7 cells in order to see if apoptotic osteocytes secrete specific soluble factors that can indirectly activate osteoclastogenesis. The conditioned medium of irradiated osteocytes (including apoptotic bodies) was not unable to increase the number of TRAP positive multinuclear osteoclasts nor was it able to increase the number and the surface area of the pits compared to the condition medium from the non-irradiated MLO-Y4 cells when seeded on a Corning plate (**Figure 5**). The inability of apoptotic osteocytes to stimulate osteoclastogenesis (#OCs + pit formation) in an indirect manner was contradictory to our hypothesis.

Because of this contradiction, we analyzed if apoptotic osteocytes activated osteoclastogenesis through direct contact. In order to demonstrate this, we adopted the co-culture method by culturing both RAW264.7 cells along with MLO-Y4 cells on a single plate and exposing the plates with various doses of radiation (no irradiation, 2Gy, 2x2Gy, 4x2Gy). We examined the osteoclastogenetic ability of the apoptotic osteocytes by comparing the number of TRAP positive osteoclasts and the area of pit formation on Corning plates. Similar to the results on the conditioned medium, irradiated MLO-Y4 cells not only failed to increase the area of pit formation on the corning plate, but also failed to increase the differentiation of RAW264.7 cells into TRAP

positive osteoclasts, with the exception of high irradiation doses (4x2Gy), compared to the plate seeded with only RAW264.7 cells (**Figure 6**).

These results show that irradiated MLO-Y4 cells that undergo apoptosis do not secrete any soluble factors nor does it express on its cell surface or through direct contact that initiate or propagate the differentiation of RAW264.7 cells into functioning multinuclear TRAP positive osteoclasts.

We now focused our attention on what other factors or cells in the bone microenvironment could be responsible for the increase in osteoclastogenesis due to radiation induced bone loss. Although the bone marrow consists of a heterogeneous population of hematopoietic stem cells, radiation is well known to suppress and change drastically the biologic features of the bone marrow environment. We collected the bone marrow from 14 week old male NOD-SCID mice and irradiated the cells with faxitron X-ray at a dose of 10 Gy. The conditioned media collected from the irradiated bone marrow cells were then added to the RAW cells to see if it can enhance osteoclastogenesis. The conditioned media from the irradiated bone marrow cells could not increase the number of osteoclasts nor could it increase the pit-formation compared to the non-irradiated conditioned media (**Figure 5**). Interestingly, irradiated bone marrow cells co-cultured with the RAW cells significantly stimulated osteoclastogenesis (#OCs + pit formation) in all varying doses of radiation (**Figure 6**).

Since we irradiated the RAW264.7 cells as well as the MLO-Y4 cells and the bone marrow cells, we wanted to elucidate whether or not radiation could directly stimulate the RAW cells into osteoclasts or could it disrupt its biologic integrity. We directly irradiated the RAW264.7 cells with various doses of radiation (no irradiation, 1x2Gy, 2x2Gy, 4x2Gy) and cultured the cells on a corning well plate to analyze the pit resorption activity. Direct irradiation of the RAW cells increased the differentiation to osteoclast and hence increased area of pit formation (**Figure 7**). This leads to a conclusion that, despite the exact mechanisms are unclear, direct effects of radiation on osteoclast progenitors and/or indirect effects on bone marrow cells may stimulate osteoclastogenesis and could serve as the primary player that drives the radiation-induced bone loss (**Figure 8**).

- ❖ Completion of this task will reveal whether radiation-induced apoptosis of osteocytes can induce osteoclastogenesis. *Cells lines used: RAW264.7, MLO-Y4, MC3T3-E1*

Task3: Test effects of single limb exposure (distal half of the femur, entire tibia) of 2Gy (months 6-10).

- a. Perform histomorphometry (OC, OT, OB counting) and in vivo bone imaging (months 6-10)

20 week old female C57Bl/6 mice (n=30) were used in this experiment. The mice were divided into 2 groups. One group (group A, n=15) was imaged twice by microCT during the experiment that included a baseline microCT that was given 2 days prior to the experimental radiation, and after 7 days a second microCT was scanned. While the second group (group B, n=15) was scanned only after 7 days after the experimental radiation. The second group was established in order to normalize the effects caused by the radiation from the microCT. Mice from all groups were anesthetized with isoflurane and irradiated on the left limb with a nominal surface dose of 2Gy using a therapeutic X-ray Machine (XRAD 320 Precision) operating at an effective dose rate of 1.5946 Gy/min. Following the last in-vivo microCT scan, mice were euthanized by cervical dislocation and blood was collected and the tibia and femur were isolated for bone histomorphometry along with the vital organs.

The bone micro-architecture of the proximal tibiae and the distal femur was assessed in-vivo using Scanco Viva 40 micro-CT system. The scanning parameters for in vivo microCT imaging were 10 μ m voxel size, 200ms integration time and 1000 projection per 180°. Focal radiation to the hind limbs was well tolerated by the mice. None of them experienced any clinically significant complications from the procedure. Body weights were recorded on the beginning and the end of the experiment, and there was no evidence of systemic ill effects in irradiated mice.

To investigate whether exposure to 2Gy of radiation might have an effect on bone architecture, we compared the bone architecture of the proximal tibia in before and after the irradiation of the left limb by using in-vivo micro-CT (Group A). Analysis of the bone volume showed a significant loss (-28.2%, p=0.0001, paired T-test) of trabecular bone (trabecular BV/TV) in the irradiated limbs compared to the baseline bone volume (**Figure 9**). Trabecular thickness as well as trabecular number and trabecular spacing did not significantly change over time in the irradiated tibia. The irradiated tibia lost Conn.D (-44.3%, p<0.001) compared to baseline, while SMI failed to increase to a significant amount. The contralateral limb of this group showed a decrease in trabecular bone volume (BV/TV: -11.1%, p=0.0163) and trabecular thickness (-3.76%, p=0.0056) but to a much lesser degree. We also compared the difference of the bone architecture between the irradiated limb and the contralateral non-irradiated limb (Group B). Analysis of the bone volume by micro-CT imaging of the tibiae showed that the 2Gy of radiation decreased trabecular bone volume by 21.36% in the irradiated tibia compared to the non-irradiated control tibia. This decrease was also accompanied by 34.24% decrease of Conn.D and a 7.71% increase in SMI, which are highly indicative of decreased both bone quality and quantity (**Figure 10**).

To determine if the decrease of bone volume and the disruption of bone micro-architecture were due to increase of osteoclastic bone resorption or decrease of osteoblastic bone formation, we analyzed histological parameters and compared the number of TRAP positive osteoclasts per bone

surface for both the irradiated and non-irradiated contra-lateral limb and also measured quantitatively with dynamic bone parameters for new bone formation and mineralization from trabecular bone within the proximal tibia and the distal femur metaphysis, respectively, from mice irradiated with 2 Gy radiation. The number of TRAP positive osteoclasts per unit bone surface was significantly higher (30% higher) for the irradiated limb compared to the contra-lateral non-irradiated limb (**Figure 11**). The dynamic bone formation parameters indicated that there weren't any statistical significant difference between mineralizing surface per Bone Surface (MS/BS) and Bone Formation Rate (BFR) for the irradiated limb compared to the non-irradiated limb, except for Mineral Apposition Rate (MAR), but there was some modest tendency leaning towards decreased bone formation (**Figure 12**). Taken together, these results show that increase in bone resorption by osteoclasts is the predominant contributing factor responsible for the decrease of bone quantity induced by ionizing radiation.

b. Determine apoptosis of bone cells (OT, OB & OC) by quantifying TUNEL staining.

The method we used to detect late phase apoptosis is terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. It is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage. TUNEL assay on paraffin-embedded tissue sections was performed as recommended by the manufacturer. The effect of radiation was noticeable when you compare the bone marrow cellularity by counting the number of adipocytes in the bone marrow and the level of bone marrow cell apoptosis detected by TUNEL staining.

The irradiated bone had a significantly increased number of adipocytes in the bone marrow compared to the contralateral control ($p = 0.02$) (**Figure 13B**). Corresponding to the decreased cellularity and increase of adipocytes in the irradiated bone marrow, the irradiated limb did not show any significant increase of TUNEL positive apoptotic bone marrow cells (**Figure 13C**). The percentage of TUNEL positive osteocytes in the trabecular bone was significantly higher in the irradiated bone compared to the contralateral control ($p < 0.05$). Although to a lesser degree and statistically insignificant, there was a slightly higher percentage of TUNEL positive osteocytes in the cortical bone as well (**Figure 13D**).

Empty lacunae are indicative of osteocyte death. There by including the number of empty lacunae to the total number of TUNEL positive osteocytes, we assumed that we could get a more accurate measurement of the exact percentage of apoptotic osteocytes. The irradiated bone had a no significant difference in

percentage of empty lacunae compared to control per se, and when added to the number of TUNEL positive osteocytes, the statistical significance was altered (**Figure 13E**).

Animal Usage: 10 balb/c mice,

- ❖ Completion of this task will reveal whether osteocyte apoptosis is associated with induced osteoclastic bone resorption.

Task4: Determine if blocking osteocyte apoptosis will block the bone loss associated with radiation (months 10-16).

- a. Single limb exposure of 2Gy (month 10).

During this period, we have completed an in-vivo pilot experiment applying the single limb irradiation model to young 4 to 5 week old male Balb c nude mice. The purpose of this experiment is to verify the effects of radiation induced bone loss are similar on different strains of mice (C57Bl/6 mice versus Balb c nu/nu) and on different age groups (20 week old versus 4-5 week old). The mice strain and the age group that we are planning use for the prostate cancer metastasis model are going to be 4-5 week old Balb c nu/nu mice. The experimental set up will be the same as the previous experiment using C57Bl/6 mice, but the only difference will be the age group and the strain. We have completed the in-vivo experiment and obtained all the necessary imaging (BMD, microCT), and collected the all tissue for histologic analysis. All the data for this experiment are currently under analysis and the results are pending.

- b. Treat mice with two apoptosis inhibitors: **i)** A caspase inhibitor that has been used in mice to inhibit osteocyte apoptosis induced by bone over-loading; and **ii)** a bisphosphonate that inhibit osteocytes apoptosis, but that does not affect osteoclasts (IG9402). Zoledronic acid will be used as positive control for inhibition of apoptosis and also inhibition of resorption (month 10).

This task has not yet been completed. Due to the fact we have found no convincing evidence in-vitro that radiation induced osteocyte apoptosis is the cause of osteoclastogenesis and bone loss. We are currently discussing with our peers and collaborators on the legitimacy of this experiment. We also need to obtain quantitative evidence that radiation induces bone loss on immunodeficient 5 to 6 week old balb/c nude mice similar to what we have observed on the immunocompetent 20 week old C57Bl/6 mice prior to initiating this task. We are planning to postpone this experiment to the second year.

- c. Perform in vivo bone imaging and histomorphometry and assess apoptosis of bone cells (osteocyte, osteoblast and osteoclast) by quantifying TUNEL staining (months 10-14).

Animal Usage: 40 balb/c mice,

- ❖ Completion of this task will reveal (1) whether radiation-induced bone loss is mediated via osteocytes, and (2) what treatment is most effective in inhibiting the radiation-induced bone loss.

Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido

Task5: Image calvarial osteocytes in real-time after single dose exposure of 2 Gy (months 6-12).

A single dose of 2Gy will be given to the calvariae of transgenic mice, in which GFP is specifically expressed in osteocytes (DMP1-EGFP mice).

- a. Develop intravital multiphoton fluorescence microscopy (IVFM) for real-time imaging of osteocytes in calvariae of transgenic mice using i) GFP to identify osteocytes; ii) Hoechst to identify apoptotic cells; iii) Texas red to identify vasculature. A time course of morphological changes will be monitored in the same animals at every 2 days during the first week post radiation and then weekly for up to 4 weeks. Animals will be euthanized 7, 14, 21 or 28 days after initiation of the experiments and we will (month 6-10)
 - Quantify in vivo + ex vivo osteocyte apoptosis, determined as green cells that contain Hoechst nuclei that are condensed (apoptotic). (month 6-7).

We have not been able to complete this task. The reason for this is that the technician who was supposed to perform the real time intravital multiphoton fluorescence microscopy (IVFM) just left the university when we wanted to start the experiments. At that time, we thought it would be best to postpone this task to the second year, and start working on other task.

- b. Perform histomorphometry (OC, OT, OB counting) and in vivo bone imaging (months 10-14)
 - Ex vivo determination of apoptosis by quantifying TUNEL staining (months 10-14).

We will postpone this task to the second year due to the situation stated above.

Animal Usage: 24 transgenic DMP1-GFP mice, in which GFP is specifically expressed in osteocytes.

- ❖ Completion of this task will reveal the time-dependent effects of a single dose of radiation of 2Gy on osteocyte apoptosis. *Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido, Dr. Carlesso*

Task6: Real-time imaging of the effects of apoptosis inhibitors on osteocytes in the calvariae (months 12-15).

- a. Set up radiation model of real-time imaging as described in Task5, and treat mice with apoptosis and bone resorption inhibitors as described in Task4 (month 12).

We have not been able to complete this task. The reason for this is that the technician who was supposed to perform the real time intravital multiphoton fluorescence microscopy (IVFM) just left the university when we wanted to start the experiments. At that time, we thought it would be best to postpone this task to the second year, and start working on other task.

- b. Assess the effects of treatment on osteocyte apoptosis with IVFM, histomorphometry, by quantifying TUNEL staining (months 12-15).

We will postpone this task to the second year due to the situation stated above.

Animal Usage: 40 balb/c mice

- ❖ Completion of this task will show a time-course of the inhibitory effects of treatments on osteocyte apoptosis.

Collaborators/consultants: Dr. Mendonca, Dr. Bateman, Dr. Bellido, Dr. Carlesso

Task7: Determine if radiation induces growth of prostate cancer bone metastases with a lytic (PC-3) or mixed (C4-2B) phenotype, and whether this will be inhibited by osteocyte apoptosis blockers (months 5-18).

- a. Intracardiac injection of C4-2B/IFP or PC-3/IFP prostate cancer cells, 1 week after single limb exposure of 2Gy (month 10).

During this period, we have currently received PC-3M-Pro4/Luc2 prostate cancer cells from Dr. Gabri van der Pluijm lab (LUMC, Leiden, Netherlands). The PC-3M-Pro4 was generated (18,19) from PC-3M (subclone of regular PC-3 prostate cancer cell line) by injecting PC-3M cells into athymic mouse prostates and selecting for variants with increasing metastatic potential by several rounds of reinjecting cells from xenograft tumors back into the mouse prostate. Dr. Gabri van der Pluijm's lab had transfected the PC-3M-Pro4 prostate cancer cells with a mammalian codon-optimized luciferase driven by a CAGGS promoter, which is up to 100x more sensitive. We have injected the PC-3M-Pro4/Luc2 cells into the left ventricle of 4-6 week old balb/c nude mice and confirmed the bioluminescence signals acquired by optical imaging with reference to the radiologic image. We also evaluated the biologic

behavior of PC-3M-Pro4/Luc2 cells and compared it with a conventional PC-3 prostate cancer cell line we had in stock. The results are currently being evaluated.

- b. Treat mice with apoptosis inhibitors *as described in task 4* (month 10).
- c. Assess tumor burden and bone loss by in vivo near-infrared imaging (Optix MX3), bone histology and histomorphometry (OC, OT, OB counting) (months 12-16)

Animal usage (cell line used): 40 balb/c nu/nu mice (PC-3 pcc) + 40 SCID mice (C4-2B pcc)

- ❖ Completion of this task will reveal (1) whether radiation induces formation and growth prostate cancer bone metastases with lytic or mixed phenotype, and (2) what treatment is most effective in inhibiting it.

Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido

Task8: Determine if radiation induces growth of blastic prostate cancer bone metastases, and whether this will be inhibited by osteocyte apoptosis blockers (months 12-18).

- a. Intra-bone injection with LuCaP 23.1 (AR+, PSA+) prostate cancer cells in both tibiae (month 12).
- b. Single limb exposure with 2 Gy (month 12).
- c. Treat mice with apoptosis inhibitors *as described in task 4* (month 12).
- d. Assess tumor burden and bone loss by radiographic and histomorphometric analyses (months 12-18)

Animal Usage (cell line used): 40 SCID mice (LuCaP23.1 prostate cancer cells)

- ❖ Completion of this task will reveal (1) whether radiation induces growth of prostate cancer cells that yield a blastic phenotype in bone, and (2) what treatment is most effective in inhibiting it.

Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido.

Task9: Image prostate cancer cell metastasis to bone in real-time after single dose exposure (months 10-18).

- a. Set up radiation model of real-time imaging as described in Task5,
- b. Intracardiac injection of PC-3/GFP pcc, 1 week after 2Gy exposure of calvarium (month 14)
- c. Treat mice with apoptosis and bone resorption inhibitors as described in Task4 (month 10).

d. Assess whether treatments affected the number of prostate cancer cells that have metastasized to bone using IVFM for real time imaging, histology and histomorphometry and TUNEL staining (months 14-18).

Animal Usage (cell line used): 40 transgenic balb/c nu/nu mice (PC-3/GFP prostate cancer cells)

- ❖ Completion of this task will reveal, in real-time, (1) whether radiation induces metastasis of prostate cancer cells to bone, and (2) at what time treatment is most effective. *Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido*

Task10: Write a review of literature (months 12-18)

*Completion of this task will result in peer-reviewed publication in a high impact journal

Task11. Prepare manuscript (months 18-24)

*Completion of this task will result in peer-reviewed publication in a high impact journal.

Site of performance

Indiana University – School of Medicine; dept. of Medicine – div. of Endocrinology

Walther Hall – R3, C644; 980 W. Walnut Street; Indianapolis, IN, 46202

In this proposal, we have allowed 4 months (months 2-5) for regulatory and approval review of all the animal studies.

KEY RESEARCH ACCOMPLISHMENTS

- While a radiation dose of 10Gy consistently induced apoptosis in MLO-Y4 osteocytic cells to large extend, only a marginal increase in apoptosis was observed upon radiation with 2Gy, based on the assay used.
- We found no evidence so far that irradiated osteocytes could stimulate osteoclastogenesis and osteoclastic activity either indirectly (conditioned medium) or directly (co-culture).
- Instead of apoptotic osteocytes irradiated bone marrow cells appear to be responsible for the stimulation of osteoclastic activity.
- In the absence of other cell types, irradiation of RAW 264.7 monocytes/osteoclast precursors already appeared to stimulate osteoclastic activity.
- The μ CT data shows that single limb irradiation with a dose of 2 Gy results in loss of cancellous bone, decreased connectivity density, degree of anisotropy and bone surface, and increased trabecular separation in the metaphysis of the proximal tibia. Decreased trabecular number and increased trabecular separation in the metaphysis of the distal femoral
- Single limb irradiation with a dose of 2 Gy results in increased number of osteoclasts per bone surface, but a significant increase in osteocyte apoptosis was not detected
- The dynamic bone formation parameters indicated that there weren't any significant difference between mineralizing surface per Bone Surface (MS/BS) and Bone Formation Rate (BFR) for the irradiated limb compared to the non-irradiated limb. These results suggest that increase in bone resorption by osteoclasts is the predominant contributing factor responsible for the decrease of bone quantity induced by ionizing radiation.
- Alternatively, direct effects of radiation on osteoclast progenitors and/or indirect effects on bone marrow cells may stimulate osteoclastogenesis and drive radiation-induced bone loss.

REPORTABLE OUTCOMES

Poster Presentations

Jeroen T Buijs, Laura Tedeschi, Marc S Mendonca, Teresita Bellido, Khalid S Mohammad, Theresa A Guise. *Radiation-Induced Osteoclastogenesis in Bone Loss: Role of the Osteocyte*. American Society for Bone and Mineral Research 2010 Annual Meeting. Toronto, ON, Canada. 2010.

JT Buijs, A Scheidler, L Tedeschi, SK John, X Peng, DR Lane, H Chin-Sinex, TA Bateman, T Bellido, MS Mendonca, KS Mohammad, TA Guise. *Radiation-Induced Bone Loss: Role of the Osteocyte*. . American Society for Bone and Mineral Research 2011 Annual Meeting. San Diego, CA, USA. 2011.

CONCLUSIONS

The in vitro effects of radiation on osteocyte apoptosis (as compared to osteoblasts) have been evaluated. It appears that despite the variation between the assays utilized to detect apoptosis (trypan blue dye exclusion, Annexin V/PI staining and Caspase-3 staining) high dose [10Gy] radiation induces significant apoptosis in MLO-Y4 cells at later time points [48 hours and 7 days]. Other bone cell lines (MC3T3-E1 and UMR-106-01) tested was also considerably affected by high dose radiation. We also saw if the apoptotic osteocytes can directly or indirectly stimulate osteoclastogenesis. In this experiment we have not found any evidence that irradiated or non-irradiated osteocytes secrete soluble factors that stimulate osteoclast formation or did we found any evidence which demonstrates that the irradiated osteocytes stimulate osteoclast formation through direct contact. But when the osteoclast precursors are co-cultured with bone marrow cells, there was significant increase in osteoclast formation and pit formation. This leads to the fact that irradiation of bone marrow cells could be responsible for the bone loss in radiation induced bone loss. Furthermore, we have found that irradiation of RAW cells can directly stimulate osteoclastogenesis and can stimulate pit formation. Taken together, we can conclude that direct irradiation of osteoclast precursor cells and some undetermined soluble factor or cells could be responsible for the activation of osteoclast formation and not the apoptotic osteocytes. The in-vivo single limb irradiation model confirms the idea that radiation significantly promotes bone loss and decreases bone quality, and it achieve this mainly by increasing TRAP positive osteoclast formation and activity. The decrease in bone formation could be a promoting factor but to a lesser extent. There was an increased tendency of apoptosis on the trabecular bone of the irradiated limb but whether or not this phenomenon is related to the increased osteoclast activity and bone loss is inconclusive. We do have much data that needs to be analyzed and many more experiments planned ahead, but the current data that we have obtained could be enough to get a glimpse of what to expect that lies ahead and give us a path towards understanding the

mechanism of radiation induced bone loss. Understanding the mechanism of osteoclast activation and bone loss in cancer patients could potentially lead to rapid implementation of therapies that would not only improve quality of life, but could also decrease morbidity and mortality after cancer treatment.

LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

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APPENDICES

1. Abstract from Endocrine Fellows Foundation Meeting and American Society for Bone and Mineral Research 2010 Annual Meeting

Radiation-Induced Osteoclastogenesis in Bone Loss: Role of the Osteocyte

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Background: Patients with tumors in pelvic region (e.g., prostate cancer, cervical cancer) frequently receive radiation therapy to treat the primary tumor. Although effective for treatment of cancer, bystander bone may experience side effects, including osteoporosis and increased fracture risk, particularly at the hip. Mouse models demonstrated that radiation-induced bone loss occurs via increased osteoclast activation. However, the exact mechanism through which osteoclasts are activated is unclear. Osteocytes (differentiated osteoblasts surrounded by bone matrix) play a vital role in determining osteoclastic activity and limited reports indicate osteocytes to be radiosensitive. Therefore, we hypothesize that radiation-induced osteocyte apoptosis may be involved in the activation of osteoclasts and subsequent bone loss.

Methods and Results: The aim of this project was to evaluate the effects of radiation on osteocytes in vitro. The osteocyte-like cell line MLO-Y4 was used and compared to MC3T3-E1 (osteoblast progenitor) and UMR-106-01 (osteoblast-like/osteosarcoma) cell lines. Cell cultures were exposed to clinically relevant doses of radiation, and apoptosis was determined by the expression of the apoptotic markers caspase-3 and annexin-V (in combination with propidium iodide) using Flow cytometry and performing an exclusion dye method (trypan blue).

While a marginal induction of apoptosis in MLO-Y4 cells was observed with 2Gy radiation, higher doses of radiation resulted in a greater induction of apoptosis. At later time points (48hr+7d), 10Gy radiation induced expression of the apoptotic markers in 50-60% of the MLO-Y4 cells, and 20-30% of cells were trypan blue positive. Similar trends were found for UMR-106-01 and MC3T3-E1 cells.

Conclusions and significance: Higher doses of radiation results in significant induction of apoptosis in osteocytes. Further studies are underway to test whether radiation-induced bone loss is mediated via osteocytes. If so, this may lead to rapid implementation of therapeutic interventions that will reduce bone loss and decrease the risk of bone fracture in patients receiving radiation therapy.

2. Abstract from the American Society for Bone and Mineral Research 2011 Annual Meeting

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Patients with tumors in the pelvic region frequently receive radiation therapy, and as a result, bystander bone may experience adverse effects, including bone loss and increased fracture risk, particularly at the hip region. Earlier reports demonstrated that radiation-induced bone loss occurs via increased osteoclast activation in a mouse model. However, the exact mechanism through which osteoclasts are activated is unclear. Apoptosis of Osteocytes has been shown to trigger osteoclast progenitor recruitment and differentiation, and limited reports suggest that osteocytes might be radiosensitive. We hypothesized that radiation induces osteocyte apoptosis leading to increased osteoclastogenesis and subsequent bone loss.

We evaluated the effects of radiation on MLO-Y4 (osteocytes), MC3T3-E1 (osteoblast progenitor) and UMR-106-01 (osteoblast-like/osteosarcoma) cell lines. Cultures were exposed to doses of 2 to 10 Gy radiation, and cell death and apoptosis were determined by trypan blue exclusion and by caspase-3 activation and annexin-V expression using flow cytometry, respectively.

Radiation with 2Gy significantly increased the number of trypan blue positive MLO-Y4 cells up to 14.9%, 15.5% and 14.6 % after 6, 24 and 48 hours respectively (vs 5.1-7.8% at Co levels), and 10Gy further increased these numbers up to 16.0%, 19.7% and 21.4%. The increase in trypan blue positive UMR-106-01 and MC3T3-E1 cells was most prominent 24 hours after 10Gy radiation (41.5% and 42.1% respectively).

While a marginal induction of apoptosis in MLO-Y4 cells was observed with 2Gy radiation, higher doses of radiation resulted in a much greater induction of apoptosis. At later time points (48hr + 7d), 10Gy radiation induced expression of the apoptotic markers in 50-60% of the MLO-Y4 cells. Similar trends were found for UMR-106-01 and MC3T3-E1 cells.

In conclusion, our findings demonstrate that radiation increases cell death and apoptosis in MLO-Y4 osteocytic cells, in a time and dose dependent fashion. Further studies are underway to functionally test whether radiation-induced bone loss is mediated via osteocytes. This may lead to rapid implementation of therapeutic interventions that can reduce bone loss and fracture risk in patients receiving radiation therapy.

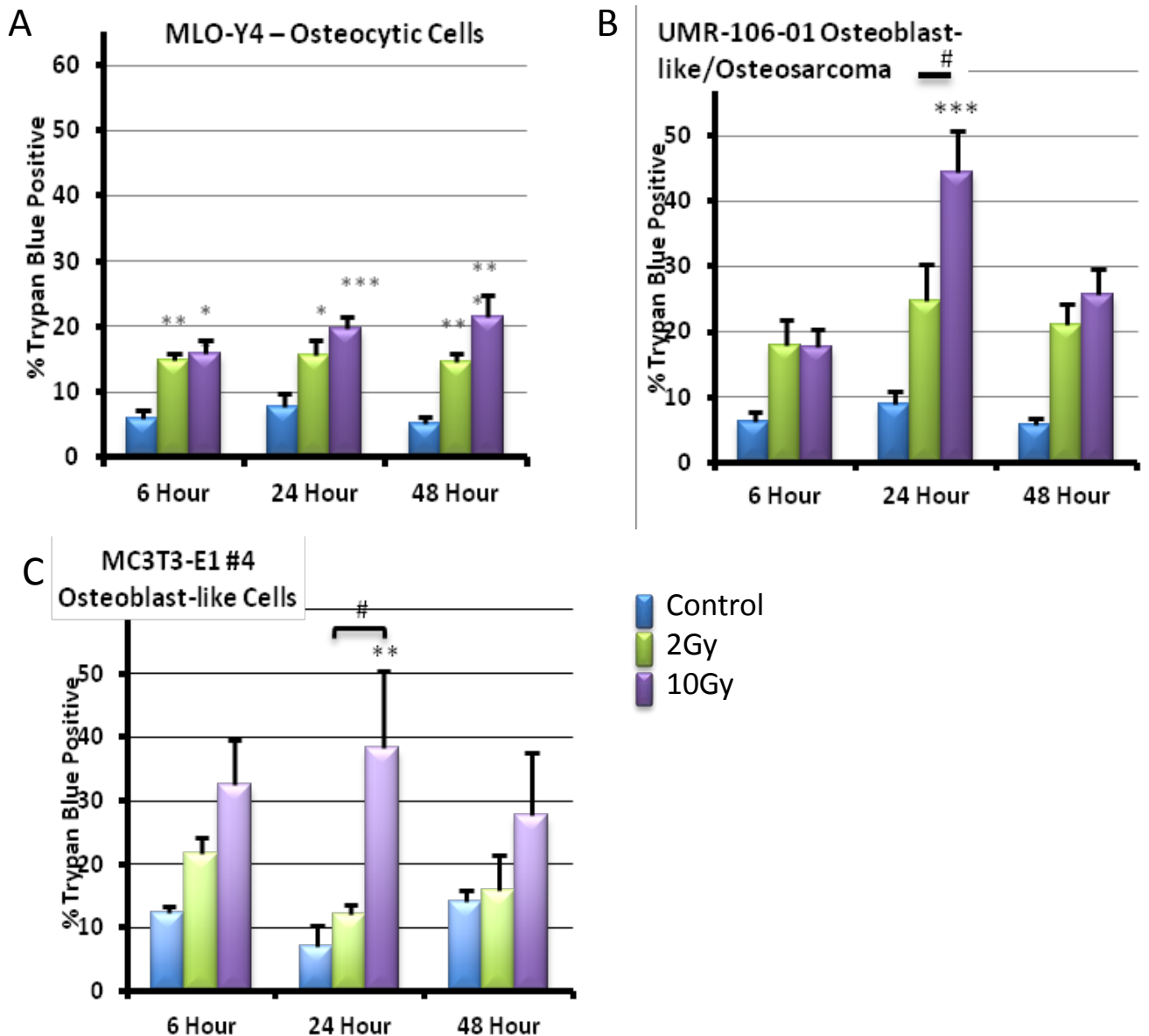


Figure 1. Cell death - Trypan Blue Dye Exclusion

Cells were exposed to a single clinically significant dose [2Gy] and single high dose [10Gy] radiation followed by analysis of apoptosis at 6, 24 and 48h using trypan blue dye exclusions analysis. MLO-Y4 (A) showed significant induction of cell death with both 2Gy and 10Gy radiation at all time points MC3T3-E1 subclone 4 (B) and UMR-106-01 (C): Dose-dependent induction of cell death, especially at 24 hours. * p<0.05, ** p<0.01, *** p<0.001 vs. Co. # p<0.05 (2Gy vs. 10Gy). Two-way ANOVA. Averages of three independent experiments performed in triplicate.

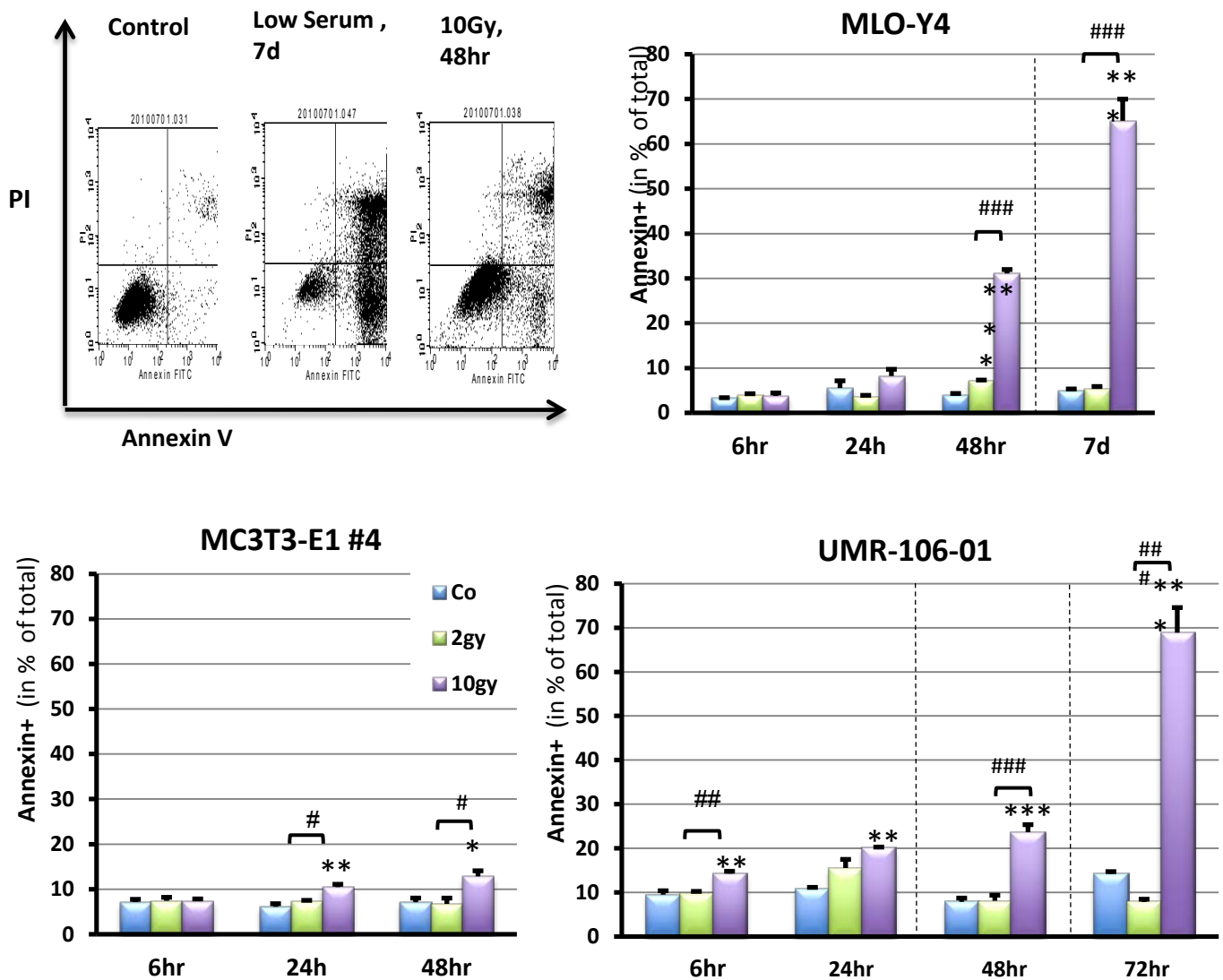


Figure 2 Flow Cytometry for Apoptotic Markers. Annexin/Propidium Iodide staining for apoptosis and necrosis respectively. Annexin – phosphatidylserine, PI – DNA in nucleus. Annexin V/PI staining shows significant increase in apoptosis at later time points with 10Gy radiation in all cell lines tested. 2Gy radiation results in a significant induction of apoptosis in MLO Y4 cells at 48 hours with Annexin V/PI analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (2Gy vs. 10Gy). Two-way ANOVA

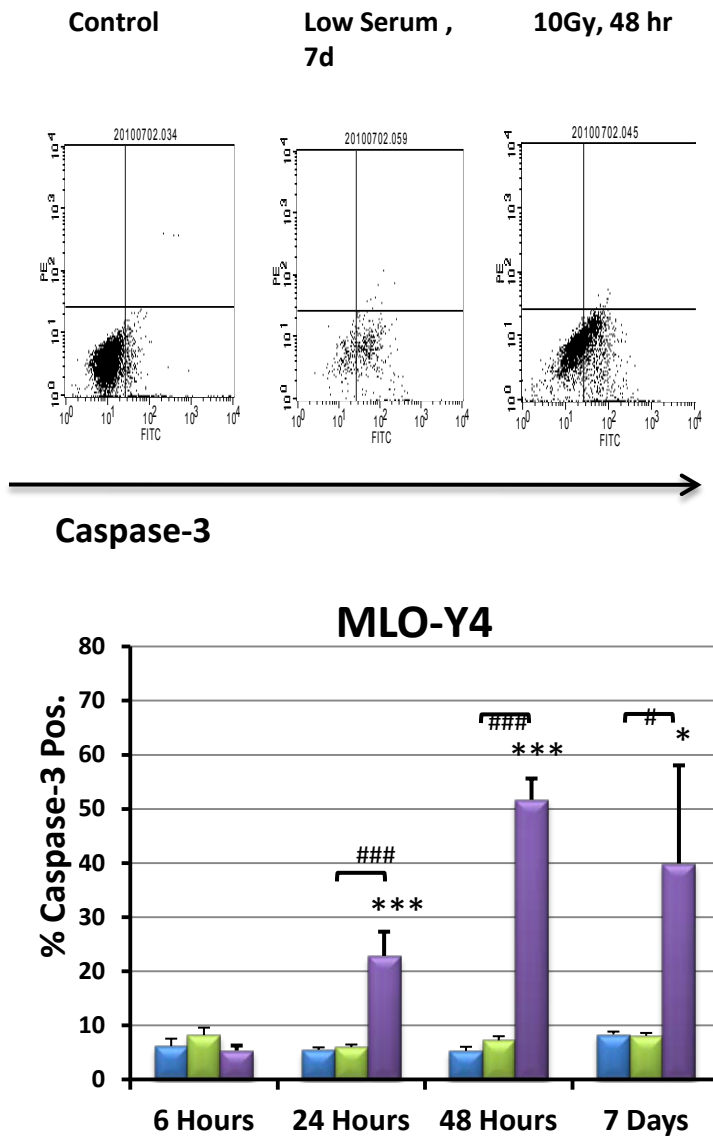


Figure 3. Flow Cytometry for Apoptotic Markers. Caspase-3 flow cytometry analysis measures the presence of Caspase-3, a intracellular marker of apoptosis. Caspase-3 Analysis of MLO Y4 cells shows significant increase in apoptotic activity with 10Gy radiation at 24, 48 hr and 7 days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; * $p < 0.05$, *** $p < 0.001$ vs. control ### $p < 0.001$ (2Gy vs. 10Gy) Two-way ANOVA

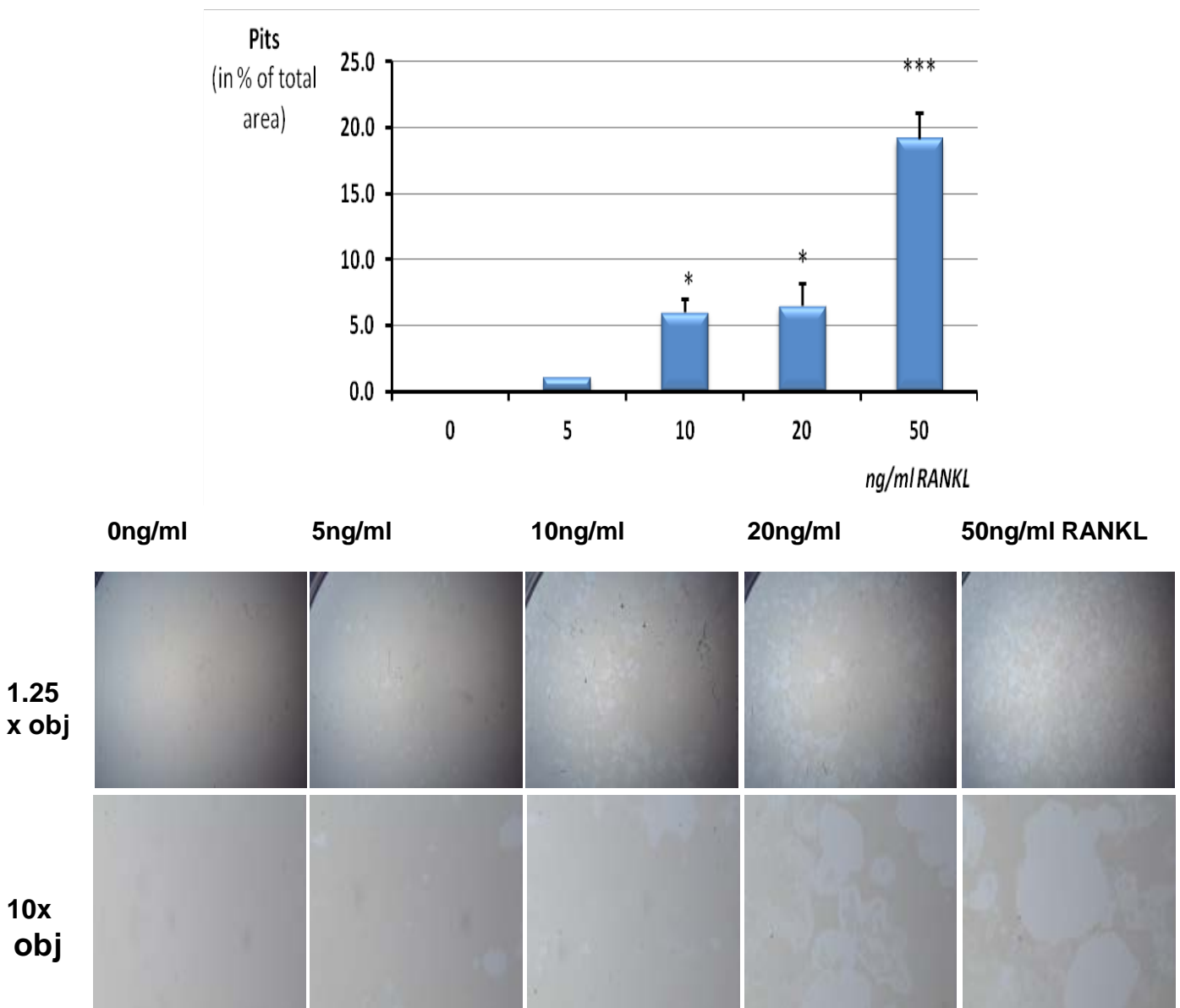


Figure 4. Osteoclast assay; 5ng/ml is the optimal concentration of RANKL required for basal stimulation of osteoclastogenesis. RAW264.7 osteoclast precursors were seeded on bone mimetic surface (Corning® plates), the medium was refreshed once, and stopped after 7 days. RANKL dose-dependently increased pit formation. RAW 264.7 cells were stimulated with 5ng/ml RANKL to establish a basal level of osteoclastogenesis. Using this concentration of RANKL, there was opportunity for more RAW cells to be stimulated as well as a little room for inhibition to be seen if it occurred.

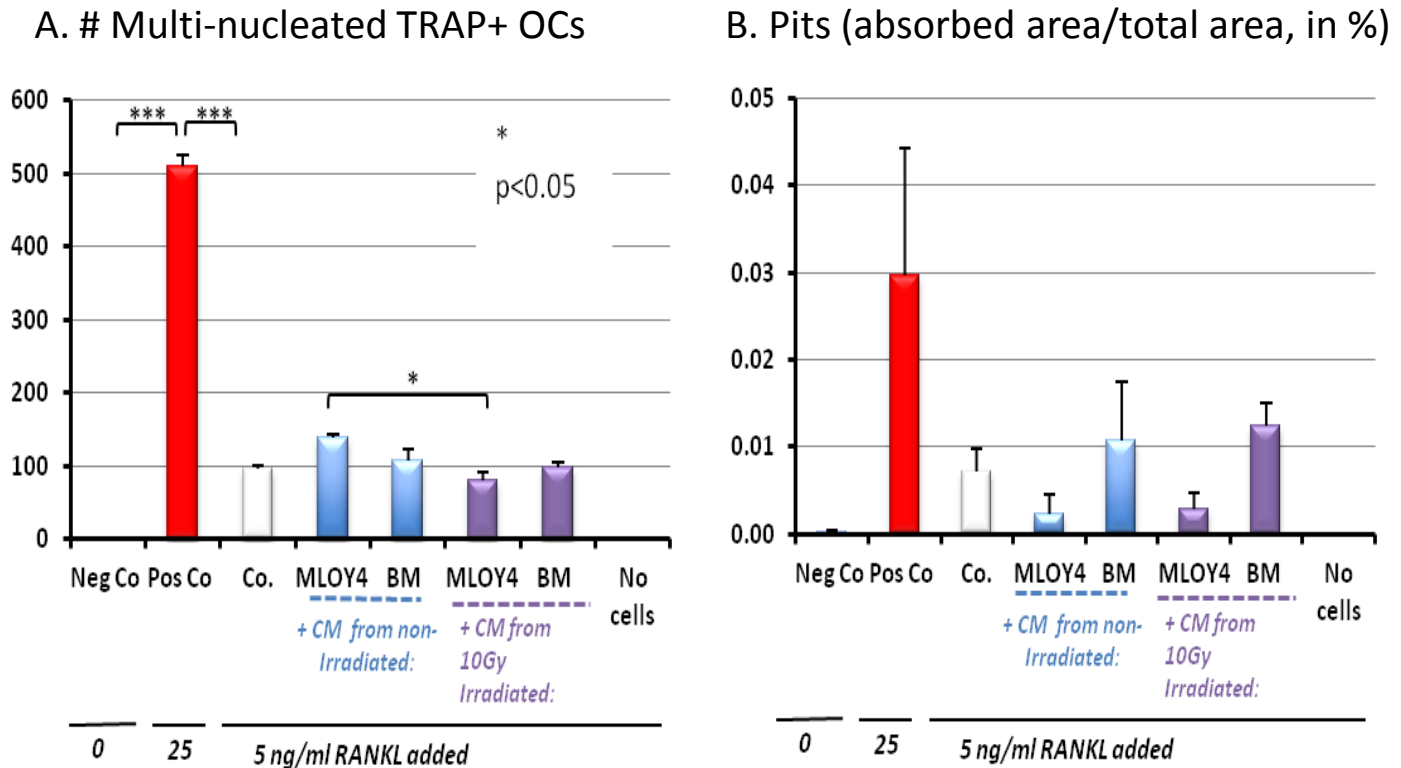
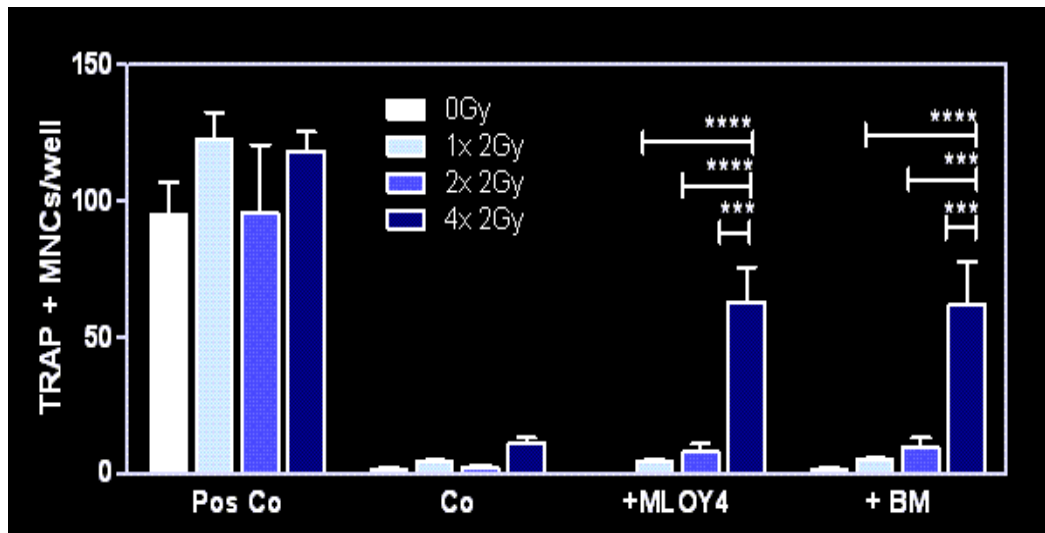


Figure 5. Osteoclast assay; indirect effect of irradiated osteocytes and bone marrow cells. Basally stimulated (with 5ng/ml of RANKL) RAW 264.7 cells were introduced to the conditioned media from irradiated (10Gy) MLO-Y4 or bone marrow cells. The number of TRAP positive multinucleated osteoclasts (A) and the percentage of pit formation (B). Soluble factors from MLO-Y4 (irradiated and non-irradiated) cells showed no significant stimulation of osteoclasts. Soluble factors released by irradiated BM cells significantly stimulated osteoclastogenesis. * $p < 0.05$, *** $p < 0.001$, One Way ANOVA.

A



B

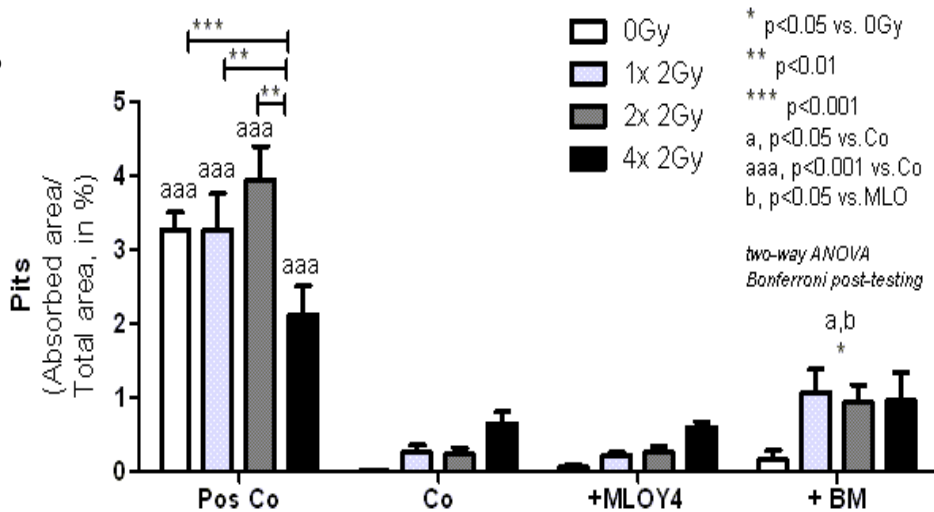


Figure 6. Osteoclast assay; direct effects of irradiated osteocytes and bone marrow cells. Basally stimulated (with 5ng/ml of RANKL) RAW 264.7 cells were co-cultured with MLO-Y4 or bone marrow cells and irradiated with different doses (0 Gy, 1x2 Gy, 2x2 Gy, 4x2 Gy) of radiation. The number of TRAP positive multinucleated osteoclasts (A) and the percentage of pit formation (B). Soluble factors from MLO-Y4 (irradiated and non-irradiated) cells showed no significant stimulation of osteoclasts. Soluble factors released by irradiated BM cells significantly stimulated osteoclastogenesis.

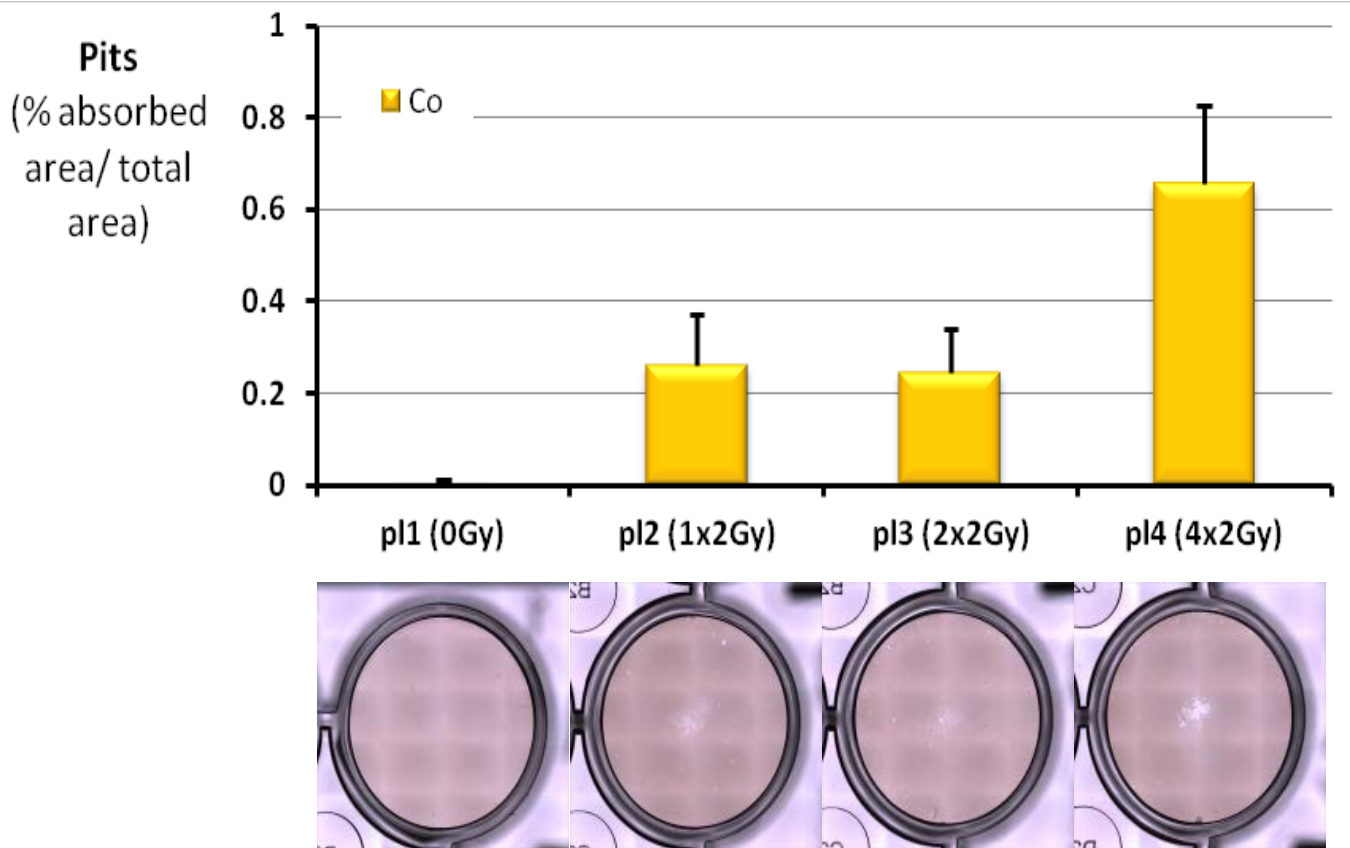


Figure 7. Osteoclast assay; direct irradiation of RAW cells stimulates osteoclastogenesis Basally stimulated (with 5ng/ml of RANKL) RAW 264.7 cells were directly irradiated with different doses (0 Gy, 1x2 Gy, 2x2 Gy, 4x2 Gy) of radiation. The percentage of absorbed area of the Corning® osteo- mimetic plates were analyzed. In the absence of other cell types, irradiation of RAW 264.7 monocytes/osteoclast precursors already appeared to stimulate osteoclastic activity.

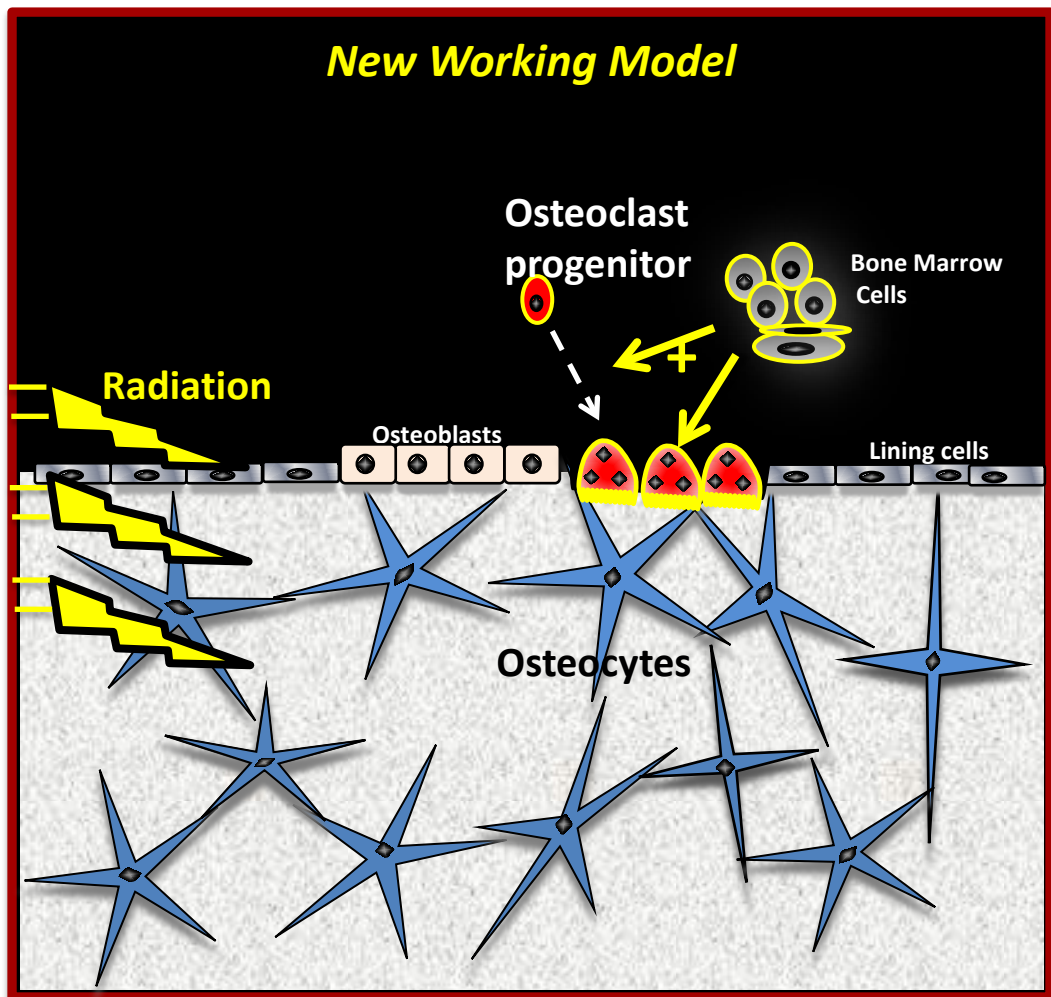
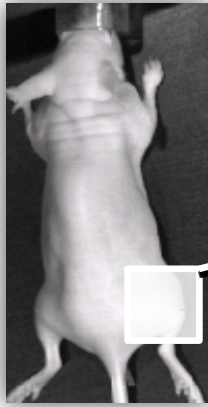
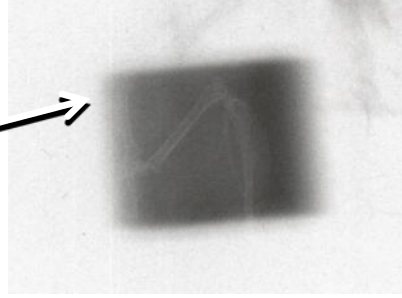


Figure 8. The new working model for radiation induced bone loss. We found no evidence so far to support that radiation-induced bone loss is mediated via osteocyte apoptosis. Alternatively, direct effects of radiation on osteoclast progenitors and/or indirect effects on bone marrow cells may stimulate osteoclastogenesis and drive radiation-induced bone loss.

A.

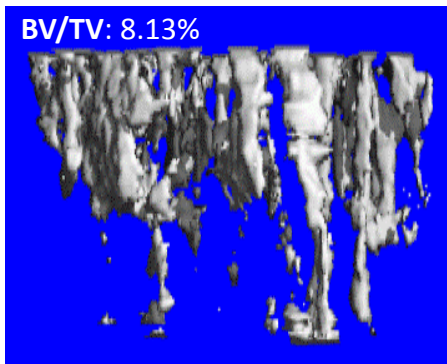


Short exposure regular X ray film



B.

Before Irradiation
(Baseline μ CT)



7days after Irradiation
(End μ CT)

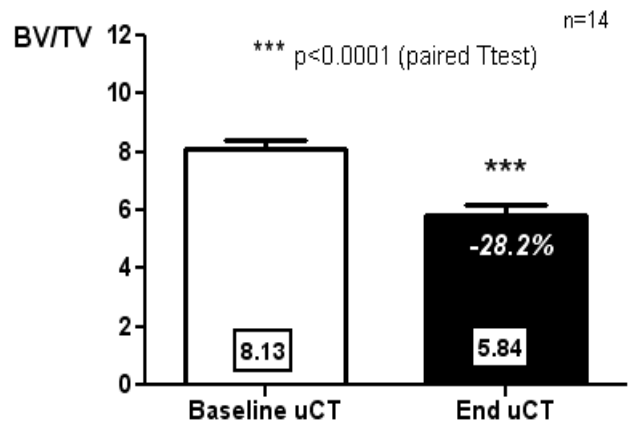
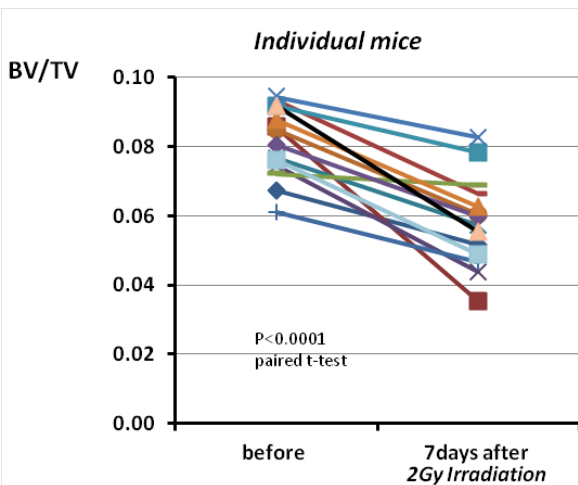
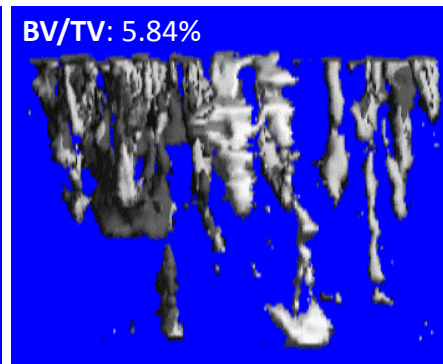


Figure 9. Single limb irradiation model (Baseline & End μ CT).

A. Using a laser-guided collimeter, mice were positioned to receive a single hind limb irradiation. The ROI is visually shown by putting a regular X-ray film underneath the mouse and irradiate for 3 seconds. (1,595 Gy/Min). **B.** μ CT images show decreased tibial trabecular bone volume following radiation (2Gy, 1.25 min 1,595 Gy/Min) in mice relative to baseline. Paired t-test.

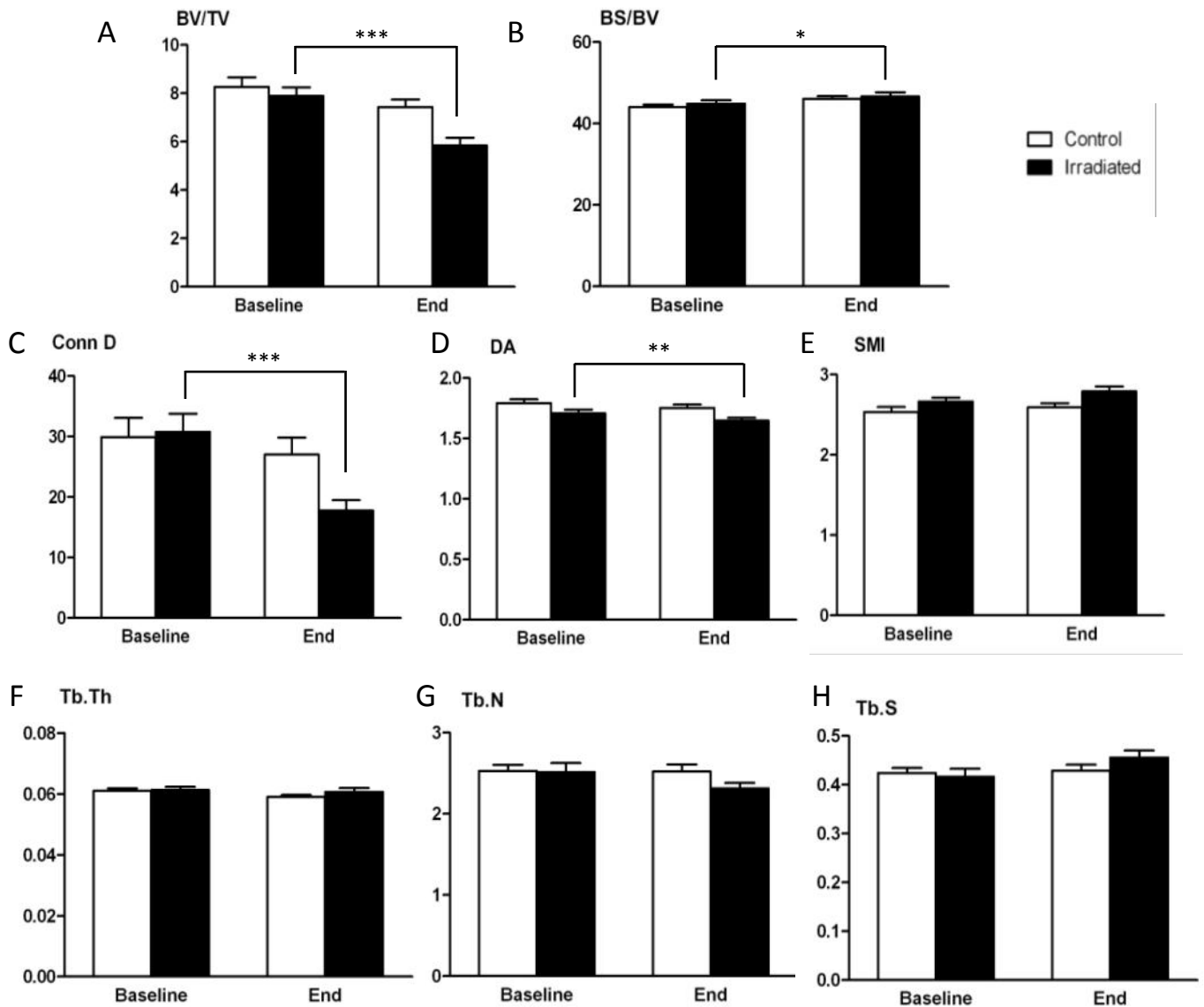
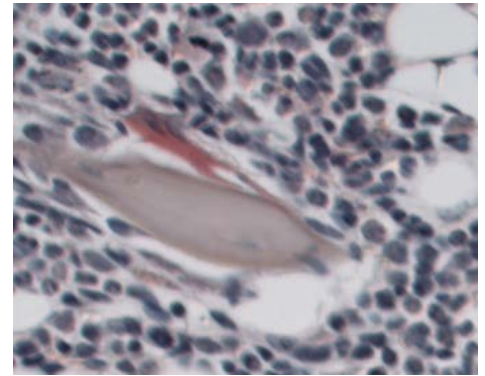
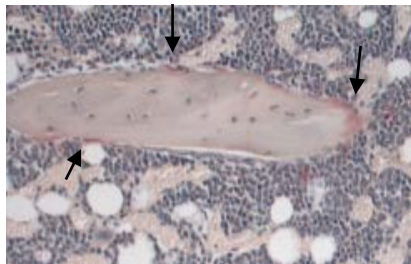
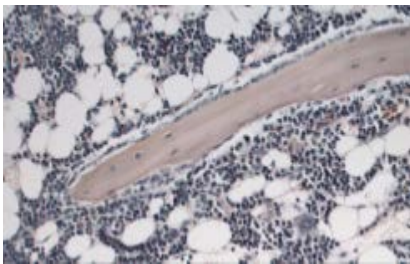
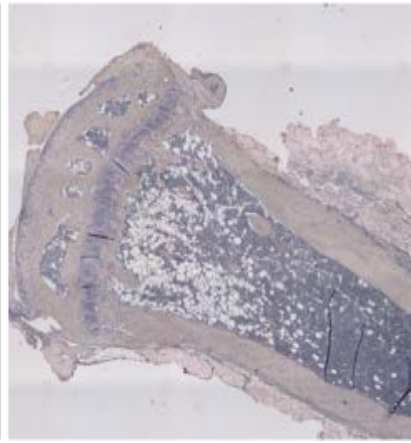
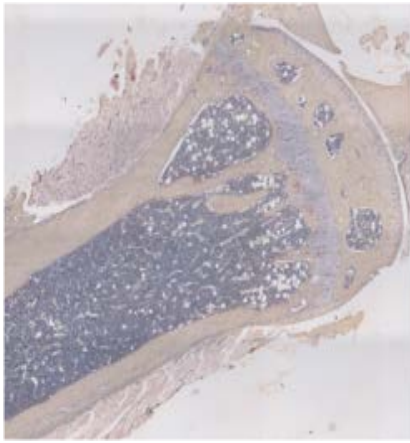


Figure 10. μ CT analysis of the bone parameters of the trabecular bone of both irradiated and non-irradiated (contralateral) tibia.

Quantitative analysis show that 2Gy of irradiation decreased the trabecular bone volume (BV/TV, fraction) (A), connective density (C), and degree of inisotropy (D), but increase of bone surface (BS/BV, fraction) (B), compared to the non-irradiated (contralateral) proximal tibiae. Paired t-test. * p < 0.05, ** p < 0.01, *** p < 0.001 using paired t-test.

Non-Irradiated
(contra-lateral)

Irradiated



Oc.N
/mm BS

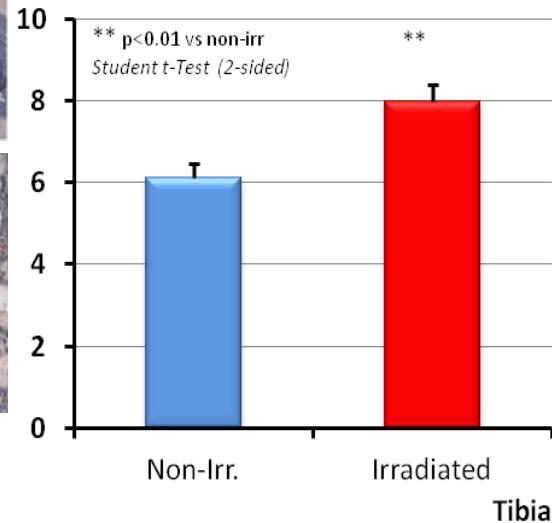


Figure 11. Histological analysis of single limb 2Gy irradiated hind limbs. (TRAP Stain). TRAP-staining on histological sections of non-irradiated (control) tibia or 2Gy irradiated tibiae of mice. There was increased number of TRAP positive osteoclasts per bone surface (OcN/mmBS) on the irradiated limb compared to the non-irradiated contralateral limb (** p<0.01 vs non-irradiated limb, student t-test).

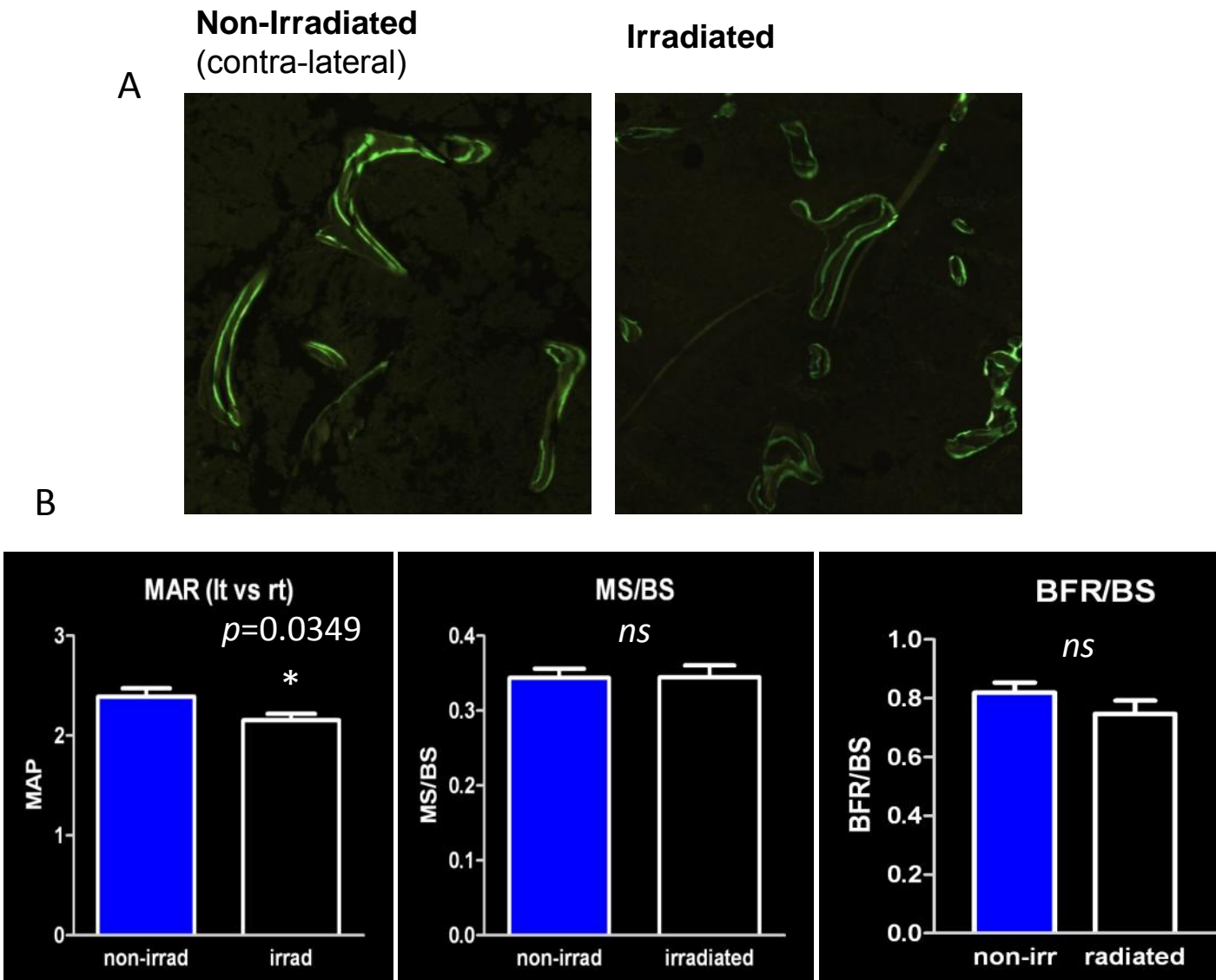


Figure 12. Quantitative measures of new bone formation and mineralization from trabecular bone at the distal femur. Representative trabecular surfaces demonstrating fluorochrome labeling. All animals were injected with two fluorochromes with a 7 day interval before tissue collection (A). No significant differences were found in mineralizing surface per bone surface (MS/BS, %) and bone formation rate per bone surface (BFR/BS, $\mu\text{m}^3/[\mu\text{m}^2 \times \text{day}]$) except for mineral apposition rate (MAR, $\mu\text{m}/\text{day}$)(B). (* $p=0.0349$ vs non-irradiated limb, Student t-test).

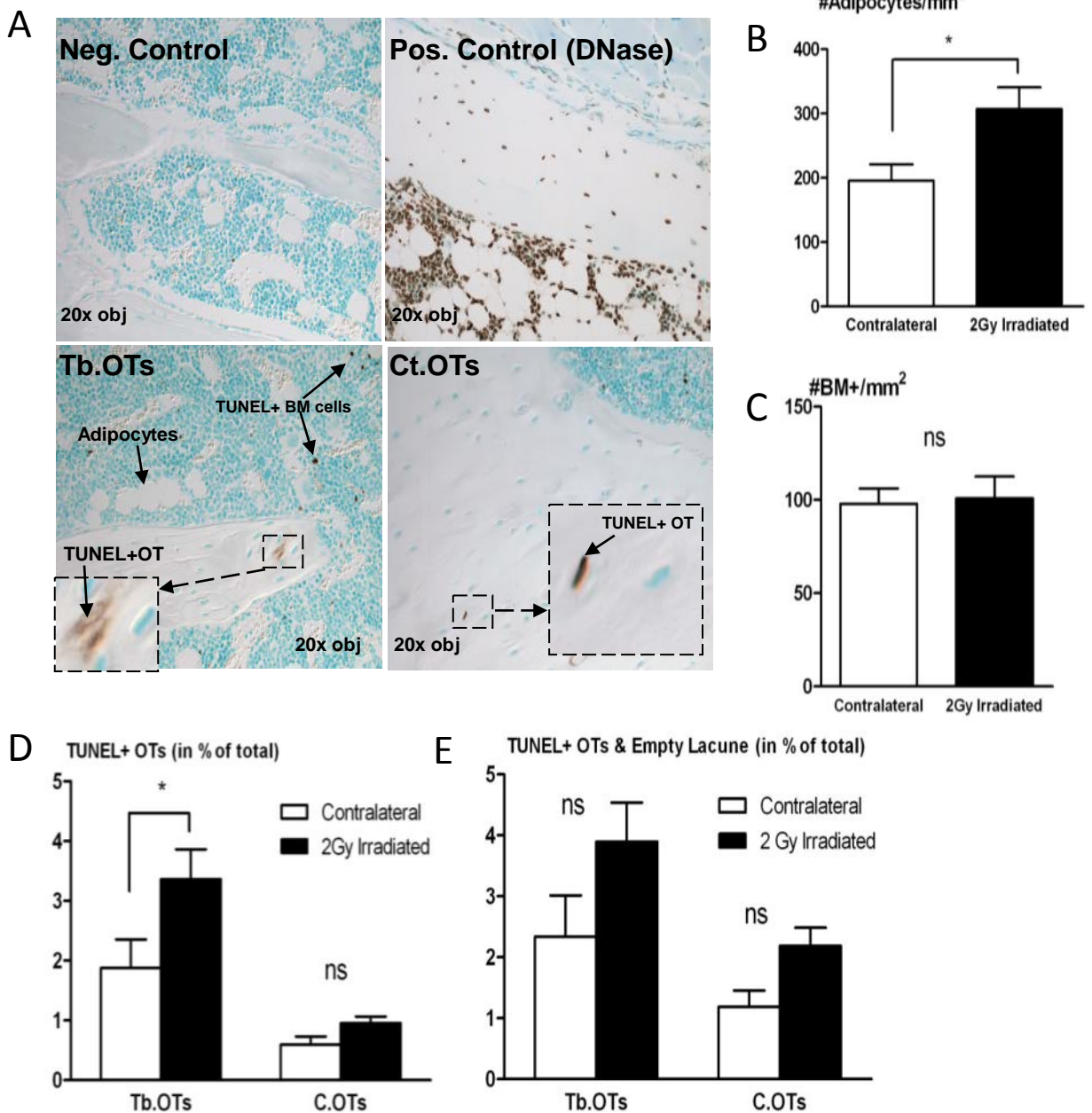


Figure 13. TUNEL analysis of apoptotic bone marrow cells and osteocytes. Representative images of negative and positive control along with sections for trabecular (Tb.) and cortical (Ct.) osteocytes (OTs) that are positive for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (A). 2Gy irradiation significantly decreased the number of adipocytes in the bone marrow cavity (B), while the percentage of TUNEL positive bone marrow cells (BM) were unaffected (C). The percentage of TUNEL positive TbOTs was increased by irradiation, however the CtOTs was unaffected (D). The incorporation of empty lacunae altered the statistical significance for the TbOTs (E). (* $p < 0.05$, Student t-test)